FEBS 15942 FEBS Letters 371 (1995) 57–60

Self-association of plasma membrane Ca^{2+}-ATPase by volume exclusion

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Received 6 July 1995

Abstract **At enzyme concentrations above 40 nM the configuration of the purified plasma membrane Ca^{2+}-ATPase is that of calmodulin-insensitive dimers. Dilution of the enzyme generates progressively higher proportions of calmodulin-sensitive monomers with lower V_{max} and Ca^{2+} sensitivity than the dimeric enzyme. Dimerization from monomeric state had not been documented before. We investigated whether concentration by volume exclusion, obtained by addition of a large molecular weight dextran to a monomeric Ca^{2+}-ATPase would elicit dimer-like behavior. Dextran induced self-association of monomers, as monitored by fluorescence energy transfer, but the Ca^{2+} sensitivity of the re-associated monomers was lower than that of the native dimers. These results suggest that the self-association reaction is structurally but not functionally reversible, and also document the existence of a hitherto unknown kinetic state of the oligomerized Ca^{2+}-ATPase, with high V_{max} but low Ca^{2+}-sensitivity.**

Key words: Ca^{2+}-ATPase; Dextran; Oligomerization; Enzyme activation

1. Introduction

The kinetics of the purified plasma membrane Ca^{2+} pump from human red blood cells was found to vary with enzyme concentration. At high enzyme concentrations (> 40 nM) Ca^{2+} activation was highly cooperative (n = 3), had a high Ca^{2+} affinity, (the apparent $K_{1/2}$ was 40–50 nM,) and the Ca^{2+}-ATPase activity was insensitive to calmodulin. At lower enzyme concentrations the properties resembled those described before for the calmodulin-sensitive Ca^{2+}-ATPase in red cell ghosts and in certain purified preparations [1,2]. The properties of the concentrated enzyme were found to be the result of enzyme self-association [1,3]. The active species of the concentrated enzyme was a dimer [4] which did bind calmodulin (one calmodulin per dimer) but was not activated by it [2,5] as were the monomers. Whereas the kinetics of the oligomerized pump is consistent with the behavior of the pump in intact cells at physiological and subphysiological $[Ca^{2+}]_i$ levels [6,7], monomeric kinetics seems to explain best the calmodulin-sensitive behavior of the pump in ghosts [8,9]. Radiation inactivation studies in ghosts indicated the existence of heterogeneous populations of pumps, including dimers [10,11]. These observations suggest that monomer-dimer configuration changes may occur in native cell membranes.

Whereas dimer to monomer conversions are easily obtained by dilution of the concentrated oligomeric enzyme, oligomerization by concentration of monomers has not yet been investigated. In the experiments presented here we examined whether concentration of monomers elicits oligomer-like kinetics. To increase the proximity between reactive molecules we added dextran (MW ≈ 60–90 kDa), an inert, large molecular weight solute, to diluted monomeric Ca^{2+}-ATPase preparations. The effects were monitored by measuring the V_{max} and Ca^{2+} sensitivity of the various enzyme configurations, in the presence and absence of dextran. Self-association of enzyme monomers in the presence of dextran was confirmed by energy transfer experiments. Addition of dextran or other large MW solutes had been shown in the past to effectively increase the activity of reactive monomers in a variety of other systems [12–15].

2. Materials and methods

Egg yolk phosphatidylcholine (P5763), CNBr-activated Sepharose 4B, and dextran (D-4751) were purchased from Sigma. Octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) was from Nikko, Japan. Coupling of bovine calmodulin to Sepharose was performed in accordance with Pharmacia instructions, as described earlier [16].

2.1. Purification of Ca^{2+}-ATPase

The Ca^{2+}-ATPase was isolated from human packed red cells purchased from local Red Cross. Red cell ghost membrane proteins were solubilized in the presence of the nonionic detergent $C_{12}E_8$, and the Ca^{2+}-ATPase was purified by calmodulin affinity chromatography as described previously [16]. The enzyme was stored at −80°C in elution buffer containing 10 mM Tris-maleate, pH 7.4, 130 mM KCl, 0.5 mM $MgCl_2$, 5 mM EGTA, 20% glycerol, 750 μM $C_{12}E_8$, 2 mM dithiothreitol, and 0.02% of sonicated suspension of egg yolk phosphatidylcholine.

2.2. Protein assay

The concentration of enzyme protein in the eluate was measured by the Bio-Rad Protein Micro-assay, based on the Bradford dye-binding procedure [17]. Bovine serum albumin was used as a standard.

2.3. Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity was determined by measurement of inorganic phosphate production, as described previously [1]. The assay was performed in a reaction mixture containing 50 mM Tris-maleate, pH 7.4, 120 mM KCl, 8 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, and $CaCl_2$, 1.01 mM for 17 μM $[Ca^{2+}]$ in Figs. 1–3 or in concentrations yielding the required free Ca^{2+} in Fig. 4. The calmodulin-dependent activation of monomers was determined in the presence of 80 nM saturating calmodulin. Appropriate aliquots of the Ca^{2+}-ATPase in the elution buffer were added to achieve the desired enzyme concentration. The concentration of $C_{12}E_8$ was kept constant at 150 μM. Total reaction volume was 100 μl. The reaction was started with 3 mM ATP and carried out for up to 30 min at 37°C. Aliquots were withdrawn at various times for colorimetric inorganic phosphate measurement [18]. Steady-state velocities were obtained from plots of inorganic phosphate production which were linear with time. Blanks were measured for each concentration of dextran to eliminate any interference of the solute with the phosphate detecting reagent used in our colorimetric assay. Fresh solution of 30% dextran (average molecular weight 60,000–90,000) was prepared daily; appropriate aliquots were added to the reaction mixture to achieve the desired final concentration.

2.4. Fluorescence energy transfer measurements

The Ca^{2+}-ATPase in erythrocyte ghost preparation was labeled with

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SSDI 0014-5793(95)00870-5

fluorescein 5'-isothiocyanate (FITC) or eosin 5-maleimide (EM) as described previously [3]. The labeled Ca^{2+}-ATPase was then purified by our standard procedure [16]. The determination of the suitability of the donor (FITC) – acceptor (EM) pair and the fluorescence measurements were as described previously [3].

3. Results and discussion

The effect of 10% dextran on the Ca^{2+}-ATPase was initially tested at two enzyme concentrations, 12 nM (diluted enzyme, about 60% monomeric) and 42 nM (concentrated enzyme, fully oligomeric), by determining the steady-state Ca^{2+}-ATPase activity at saturating free Ca^{2+} levels of 17 μM (V_{max}). As shown in Fig. 1 addition of dextran had no effect on the activity of the concentrated enzyme while it caused a twofold increase in Ca^{2+}-ATPase activity of the diluted enzyme. The dextran-induced enhancement of activity of the diluted enzyme was less in the presence of calmodulin whose binding to enzyme monomers already increased the Ca^{2+}-ATPase activity by approximately 50%.

The experiment in Fig. 2 was designed to characterize the effect of dextran over a wide range of Ca^{2+}-ATPase concentrations. The characteristic sigmoidal activation curve reached a plateau at ~40 nM enzyme in the absence of calmodulin, and at 20 nM enzyme in the presence of calmodulin, as described previously [1]. Addition of 10% dextran elevated the Ca^{2+}-ATPase activity at all enzyme concentrations at which the enzyme was not totally oligomerized. The increase in activity was largest (up to 100%) at the lowest enzyme concentrations, where monomers prevailed. The effect diminished with increasing enzyme concentration. Similar patterns were observed either in the absence or presence of calmodulin. However, the extent of the dextran-induced increase in Ca^{2+}-ATPase activity was less when calmodulin was available to enzyme monomers. This difference is shown in Fig. 2, inset, as percent of dextran-induced increase in Ca^{2+}-ATPase activity at each enzyme concentration respectively with and without calmodulin.

The concentration-dependence of the effect of dextran is reported in Fig. 3. 2% dextran effectively increased the activity of the 12 nM enzyme by 10–20%. 14% dextran (the highest concentration that could be tested) was several times more effective. The difference between the effects of 10% and 14% dextran was minimal. Similar dependence was observed in the presence and absence of calmodulin. In contrast to the diluted enzyme, the Ca^{2+}-ATPase activity of the concentrated, fully oligomerized enzyme was not increased at any dextran concentration (Fig. 3).

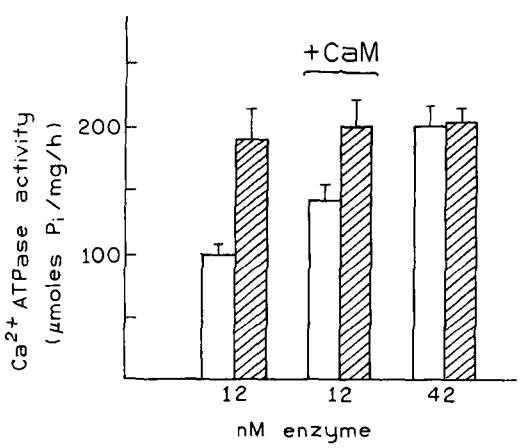

Fig. 1. Ca^{2+}-ATPase activity of the diluted (12 nM) and concentrated (42 nM) enzyme in the absence (open colums) and presence of 10% dextran (hatched columns). Diluted enzyme was assayed either with or without 80 nM calmodulin, CaM. The Ca^{2+}-ATPase activity assay was performed as described in section 2. Data are expressed as the mean ± S.E. of the mean (SEM) for 5 experiments.

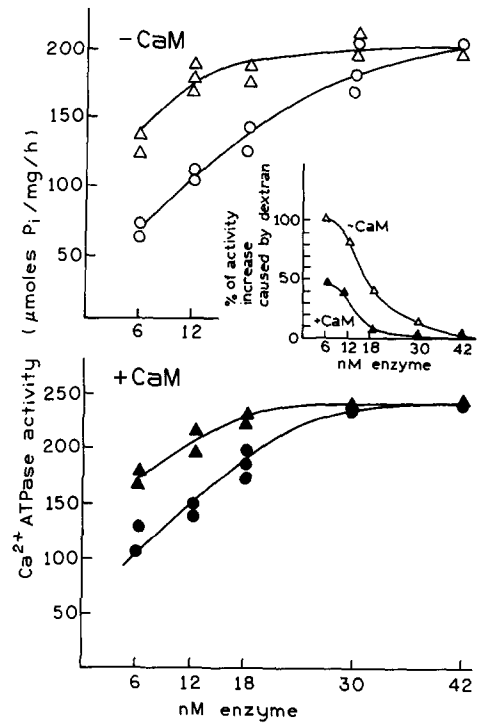

Fig. 2. Increase in Ca^{2+}-ATPase activity caused by 10% dextran (△,▲) at different enzyme concentrations. The effect was observed in the absence (−CaM, open symbols) and presence (+CaM, filled symbols) of calmodulin. Inset compares effect of dextran on enzyme activity ± CaM, as expressed in percent of control activity either with or without calmodulin. One typical experiment shown.

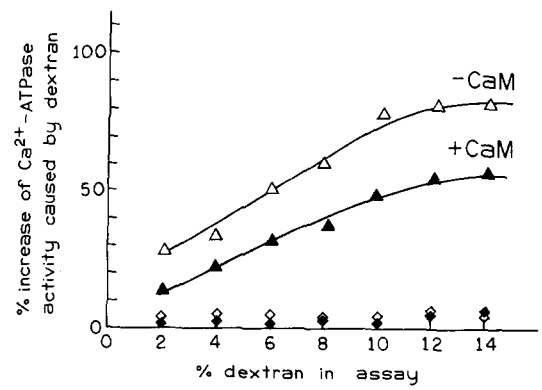

Fig. 3. Dose-dependent effect of dextran on Ca^{2+}-ATPase kinetics of the diluted 12 nM (△, ▲) and concentrated, 42 nM (◇, ◆) enzyme. The Ca^{2+}-ATPase activity is expressed as percent of control Ca^{2+}-ATPase activity which was determined in the absence of dextran for the calmodulin-independent (−CaM, open symbols) and calmodulin-dependent (+CaM, filled symbols) enzyme. For 12 nM enzyme 100% activity (control) was 100 ± 9.8 μmol P_i/mg protein/h in the absence and 146 ± 10.5 μmol P_i/mg protein/h in the presence of 80 nM calmodulin. For 42 nM enzyme the activity was 200 ± 14 and calmodulin did not stimulate it.

The results in Figs. 1–3 show that (i) dextran increases the V_{max} of the monomeric enzyme, (ii) the maximal increase in V_{max} is to the activity level of oligomers, (iii) dextran stimulates the V_{max} of calmodulin-stimulated monomers with a similar pattern as that of monomers without calmodulin, and (iv) dextran does not affect the V_{max} of oligomers. One possible interpretation of these effects of dextran is that it elicits oligomer- like behavior of the diluted enzyme by facilitating oligomerization of available monomers, both in the presence and absence of calmodulin.

To explore this possibility, we investigated whether, in addition to elevating V_{max}, dextran also shifted the Ca^{2+} sensitivity of monomers towards the lower $K_{1/2}$ of oligomers. The Ca^{2+}-ATPase activity of calmodulin-stimulated monomers (12 nM enzyme) and of oligomers (42 nM enzyme) was measured in the presence and absence of 10% dextran. The results in Fig. 4 show that dextran (i) enhanced the Ca^{2+}-ATPase activity of diluted enzyme over the whole range of Ca^{2+} concentrations studied, (ii) had no effect on the V_{max} of the concentrated enzyme, and (iii) was without significant effect on the Ca^{2+} sensitivity of both diluted and concentrated enzymes (Fig. 4, insets). Hence, on kinetic grounds at least, the effect of dextran on monomers appears to be a pure V_{max} effect.

If, as assumed, dextran acts by volume exclusion alone, then the pure V_{max} effect on monomers, together with the lack of effect on oligomers, suggests that increasing the proximity between monomers either triggers a change to a hyper-active configurational state of monomers, or else elicits self-association to oligomers, with conservation of the Ca^{2+}-sensitivity characteristics of monomers. Both alternatives correspond to hitherto unknown Ca^{2+}-ATPase configurations.

To determine whether dextran facilitated oligomerization of the Ca^{2+}-ATPase we have employed fluorescence energy transfer techniques that allowed us previously to establish that this enzyme is fully activated by self-association to oligomers [3]. The following results are shown in Fig. 5: (i) at low enzyme concentrations 10% dextran significantly increased energy

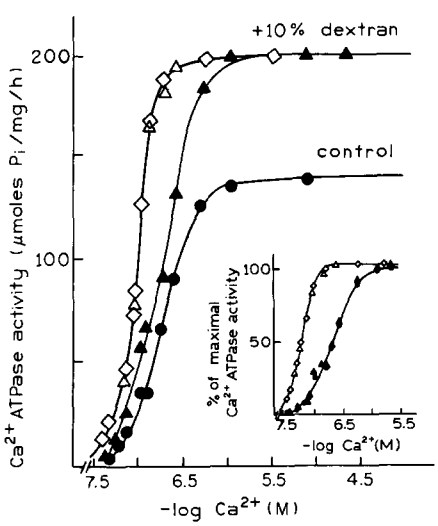

Fig. 4. Ca^{2+} concentration dependence of Ca^{2+}-ATPase activity of 12 nM enzyme plus calmodulin (● ▲) and 42 nM enzyme (◇, △) in the presence (▲, △) and absence (●, ◇) of 10% dextran. Insets show normalized data, where 100% is the maximal activity either with or without dextran.

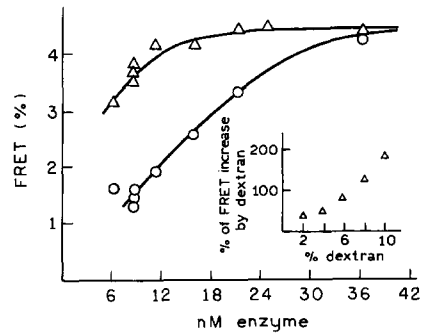

Fig. 5. Increase in fluorescence energy (FRET) caused by 10% dextran (△) relative to dextran-free controls. The efficiency of energy transfer was calculated from the decrease of FITC emission of the donor-labeled enzyme at 516 nm occurring upon addition of equal amount of EM-labeled enzyme. The measurements were performed at different enzyme concentrations indicated on the x-axis. The decrease of FITC emission was expressed in percent of the fluorescence intensity observed before addition of acceptor-labeled enzyme. The assay medium was the same as for the Ca^{2+}-ATPase activity assay, except that ATP was omitted. Inset shows concentration dependence of dextran effect as measured at 10 nM enzyme.

transfer from the donor-labeled to the acceptor-labeled enzyme, (ii) it had no effect at high enzyme concentrations, (iii) the extent of the dextran-induced increase of energy transfer was concentration-dependent, as measured between 2% and 10% dextran (Fig. 5, inset), and (iv) dextran effects on energy transfer were similar to its previously observed effects on the Ca^{2+}-ATPase activity. These results then indicate that dextran does elicit self-association of Ca^{2+}-ATPase monomers. Self-association may therefore be considered structurally a reversible reaction. However, the functional configuration of the oligomers generated by concentration of monomers differs in Ca^{2+} sensitivity from that of the native dimers eluted from the chromatography column in the original purification process. This functional irreversibility may result from modified monomer structure in the process of oligomer dissociation or from unknown effects of dextran. The fact that dextran does not seem to interfere with the high Ca^{2+} affinity of the native dimers suggests that it may not be responsible for the functional irreversibility. If dissociation does indeed generate irreversible changes in monomers which prevent the increase in their Ca^{2+} sensitivity on reassociation, these results may help explain many apparently contradictory observations on the functional properties of the Ca^{2+} pump in different preparations.

Acknowledgements: We thank Mrs. G. Roszczynska for her excellent technical assistance in the initial phase of the project. Supported by a Grant-in-Aid 92010190 from the American Heart Association (D.K.K.), by Grant GM 447130 from the National Institutes of Health (D.K.K.), and by The Wellcome Trust (V.L.L.).

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